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Exposure to elevated ozone levels differentially affects the antioxidant capacity and the redox homeostasis of two subtropical *Phaseolus vulgaris* L. varieties

Fernanda Freitas Caregnato [a,*], Rafael Calixto Bortolin [a], Armando Molina Divan Junior [b], José Cláudio Fonseca Moreira [a]

[a] Centro de Estudos em Estresse Oxidativo, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Ramiro Barcelos, 2600, Anexo, CEP 90035-003, Porto Alegre, RS, Brazil

[b] Laboratório de Bioindicação Vegetal, Centro de Ecologia, Instituto de Biociências, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves, 9500, Prédio 43411, CEP 91501-970, Porto Alegre, RS, Brazil

HIGHLIGHTS

- Higher endogenous levels of ROS protect leaves from the elevated ozone exposure.
- Insufficient leaf antioxidant capacity is related to increased ozone sensitivity.
- Early ozone-mediated ROS accumulation diminishes chlorophyll and protein content.
- Ozone sensitivity depends on the variety potential to keep the redox homeostasis.

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ABSTRACT

Ozone (O_3) has become one of the most toxic air pollutants to plants worldwide. However, investigations on O_3 impacts on crops health and productivity in South America countries are still scarce. The present study analyzed the differences on the enzymatic and non-enzymatic antioxidant system in foliar tissue of two subtropical *Phaseolus vulgaris* varieties exposed to high O_3 concentration. Both varieties were negatively impacted by the pollutant, but the responses between each variety were quite distinct. Results revealed that Irai has higher constitutive levels of reactive oxygen species (ROS) and ascorbate (AsA) concentration, but lower total thiol levels and catalase immunocontent. In this variety catalase protein concentration was increased after O_3 exposure, indicating a better cellular capacity to reduce hydrogen peroxide. On the opposite, Fepagro 26-exposed plants increased ROS generation and AsA concentration, but had the levels of total thiol content and catalase protein unchanged. Furthermore, O_3 treatment reduced the levels of chlorophylls *a* and *b*, and the relationship analysis between the chlorophyll ratio (*a*/*b*) and protein concentration were positively correlated indicating that photosynthetic apparatus is compromised, and thus probably is the biomass acquisition on Fepagro 26. Differently, O_3 treatment of Irai did not affect chlorophylls *a* and *b* content, and loss on the protein content was lower. Altogether, these data suggest that early accumulation of ROS on Fepagro 26 are associated with an insufficient leaf antioxidant capacity, which leads to cell structure disruption and impairs the photosynthesis. Irai seems to be more tolerant to O_3 toxic effects than Fepagro 26, and the observed differences on O_3 sensitivity between the two varieties are apparently based on constitutive differences involved in the maintenance of intracellular redox homeostasis.

1. Introduction

Tropospheric ozone (O_3) is a widespread secondary air pollutant which is produced through the reactions between primary pollutants (nitric oxides, sulfur oxides, carbon oxides and hydrocarbons), and sunlight (Finlayson et al., 1997). Although O_3 is produced on areas with intense primary pollution, meteorological and topographic conditions can move its precursors from these areas towards less polluted ones, such as rural zones, with detrimental effects on natural and cultivated plant species (Crutzen and Lelieveld, 2001). Over the past few years, the ground-level O_3 concentrations has become a major threat to vegetation at a

* Corresponding author. Tel.: +55 51 33085578; fax: +55 51 33085540.
E-mail address: ffcaregnato@gmail.com (F.F. Caregnato).

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global scale, and experimental studies have confirmed that exposure to elevated O_3 levels results in plant damage specially because it can suppress photosynthesis, accelerate senescence, decrease growth and reduce crop yields (Agrawal et al., 2003; Booker et al., 2009).

The threat imposed by O_3 has brought important implications for agriculture across many parts of the world and a large amount of data indicate that substantial yield losses of important crops, such as wheat, rice and soybean, arise under current O_3 concentrations (Feng et al., 2003; Fishman et al., 2010; Sarkar and Agrawal, 2010). Predictive models indicate that global surface O_3 levels are expected to rise significantly throughout the 21st century. According to Vingarzan (2004), modeling studies using the Intergovernmental Panel on Climate Change (IPCC, 2001) emission scenarios predict that the average global surface O_3 concentration is expect to be in the range of 35–48 ppb by 2040, 38–71 ppb by 2060, 41–87 by 2080 and 42–84 ppb by 2100. Such increases exceed the internationally accepted environmental criteria and can have severe implications on human health, crops and vegetation.

Numerous studies report that elevated O_3 concentrations often results in reduced photosynthesis and whole-plant growth, decreased stomatal conductance, altered antioxidant system, and accelerated senescence (Kobayashi, 1995; Calatayud and Barreno, 2004; Ashmore, 2005; Black et al., 2007; Calatayud et al., 2007; Feng et al., 2008). The effects of O_3 depend on a number of events, starting with the gas uptake through leaf stomata. In the sub-stomatal chamber, O_3 can directly react with plasma membrane through "ozonolysis" stimulating lipid peroxidation and impairing membrane fluidity, or it can be spontaneously converted into reactive oxygen species (ROS) such as superoxide radical (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) (Kanofsky and Sima, 1995; Halliwell and Gutteridge, 2007). Increased ROS levels may create a pro-oxidant environment inside the cell, resulting in proteins modification, both in their structure and activity (Iriti and Faoro, 2008). In particular, O_3 and the derived ROS can react with the exposed proteins sulphydryl groups presented on the amino acids resides, mainly cysteine, tryptophan, tyrosine, methionine and histidine, which might contribute directly to lower the overall plant vigor (Mudd et al., 1969; Foyer and Noctor, 2008). Furthermore, photosynthetic capacity and photosynthetic efficiency are decreased under high O_3 levels, which has greatly contribute to yield loss in U.S. soybean (*Glycine max*) cultivars that are particularly sensitive to O_3 (Betzelberger et al., 2012). Some studies report that O_3-mediated decline in photosynthetic pigments, chlorophylls *a* and *b*, arises as a consequence of the oxidative destruction of these molecules (Leitao et al., 2008). Changes on the levels of photosynthetic pigments modify the chlorophyll fluorescence parameters, specifically affecting the photosystem II (PSII) activity (Flowers et al., 2007).

Plant cells possess an array of antioxidants (both enzymatic and non-enzymatic) to reduce the stress elicited by the ROS. Enzymatic scavenging mechanisms in plants include important antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT). Among these enzymes, catalase plays an important role in keeping the levels of H_2O_2 under control, and some authors suggest that catalase functions as a cellular sink for H_2O_2 (Willekens et al., 1997). In contrast to superoxide and hydroxyl radical, H_2O_2 is relatively stable, and thus is capable to function as a molecular signal for cell-to-cell communication (Desikan, 2001; Vandenabeele et al., 2004). Thus, sustained increases in H_2O_2 may provoke transcriptional responses that include expression of hypersensitive response and programmed cell death genes (Vandenabeele et al., 2003). In guard cells, for example, H_2O_2 participates in the activation of membrane-localized anion channels that lead to stomatal closure (Schroeder et al., 2001; Zhang and Klessig, 2001). The importance of catalase has long been discussed and experiments performed with catalase-deficient mutant of barley (*Hordeum vulgare* L.) showed that transgenic plants present severe leaf bleaching, specifically under conditions that H_2O_2 production is favored through photorespiration (Kendall et al., 1983).

Non-enzymatic ROS scavengers include a number of compounds with high reducing power, such as the ascorbic acid and glutathione. According to several authors, the apoplastic AsA is the first line of defense against O_3-derived ROS, and O_3 sensitivity can be generally correlated to the AsA redox status of the leaf tissue (Kanofsky and Sima, 1995; Ranieri et al., 1999; Castagna and Ranieri, 2009). It has been reported that AsA levels are increased in plants challenged with O_3, suggesting that AsA plays an important role in providing resistance to oxidative stress imposed by O_3 (Menser, 1964; Sharma et al., 1997). However, some authors have recently reported that elevated apoplastic AsA levels are apparently not sufficient to explain O_3 tolerance (D'Haese et al., 2005; Dizengremel et al., 2008). Although the resulting increment on AsA levels may be a predictor of resistance to O_3 exposure (Eltayeb et al., 2006), other antioxidant molecules, and an improved cellular ability to regenerate these antioxidants could be more relevant to protect the cells against O_3 toxic effects. In plants, the physiological significance of AsA is strictly dependent on total thiol groups redox state, especially the glutathione (GSH) molecule which is the predominant non-protein thiol. Thus, the AsA–GSH system (also known as Halliwell–Asada cycle) is responsible for the maintenance of the cellular the redox homeostasis (Smirnoff, 1996; Horemans et al., 2000). In addition to GSH, thiol-redox proteins also play a important role as antioxidants and, together with GSH, are responsible for keeping the cellular redox balance (Foyer and Noctor, 2008). Analysis of total thiol content may thus be important for understanding how O_3-derived ROS changes the thiol-redox state of cellular components, such as enzymes activity modulation trough redox modifications of active site residues required for catalysis and/or ligand binding, oligomerization (Paget and Buttner, 2003).

Most studies and evidences that assess the harmful effects of O_3 on crop plants health and productivity are currently performed in countries which are experiencing a rapid increase in population and industrialization levels, such as China and India (Sarkar et al., 2010; Wang et al., 2012). Besides that, extensive studies have been conducted on the United Sates that helped to improve the scientists' knowledge about the biochemical, genetics and molecular aspects ruling O_3 toxicity in native and agronomic plants (Krupa et al., 2000; Booker et al., 2009; Betzelberger et al., 2010).

However, O_3 trend data for the Southern Hemisphere is sparse, and regional characterization is still necessary to determine the levels of O_3 observed on the industrialized southern countries (Vingarzan, 2004). Furthermore, investigations and evidences with respect to vegetation damage and human health effects of O_3 in South America countries are scarce. In Brazil, relative little information concerning the levels and the impacts of O_3 pollution on plants health and crop productivity are available and, therefore, it should be some of the urgent research priorities for the future of the country. Over the past few decades, Brazilian commodities production has expanded and the country has observed a rapid rise in exports of crops, like soybean and wheat. Crop area has expanded rapidly and is expected to continue to do so, shifting the crop cultivation to new regions of the country. This scenario has brought an increment on the production of the major cultivated crops.

According to the US DA (2010), Brazil is currently the largest world bean producer and consumer. The National Food Supply Agency (CONAB) had forecasted Brazilian dry bean production for 2010/11 at 3.45 million metric tons, up 5% when compared

to 2009, with a cultivated area estimated at 3.6 million hectares, up 1% percent from 2009. Yield was forecasted at 0.965 tons/hectare, 6% greater than 2009 and well above the 5-year average of 0.857 tons/hectare. Dry beans are cultivated in nearly every Brazilian state, but most of the production is concentrated in 8 states, which are responsible for approximately 80% of Brazil's production. Family farmers produce about 70% of total bean production.

Feng and Kobayashi (2009) conducted a quantitative meta-analysis study to assess the effects of rising O_3 concentrations on yield and yield components of major food crops (potato, barley, wheat, rice, bean and soybean) and observed that bean was the crop with the largest yield loss (19%). The authors indicate that bean is very sensitive to O_3, which is in agreement with the study of Mills et al. (2007), who ranked bean as the most sensitive among agricultural crops according to O_3 critical levels (AOT40 for 5% yield reduction).

Keeping in view that O_3 levels are increasing worldwide and Brazil is a developing country that still suffer from a general lack of information concerning the negative impacts of O_3 over the major agricultural crops, the aim of the present work was to analyze the effects of O_3 exposure on the redox homeostasis balance on leaf tissue of two Brazilian bean varieties (Fepagro 26 and Irai). These varieties were developed by the Founding State of Agricultural Research of Rio Grande do Sul (FEPAGRO-RS) and are exclusively cultivated in the southern states of Brazil. In spite of that, no information describing the physiology or the chemical profile of these varieties was done before. Thus, our laboratory decided to start the investigation on how O_3 affects the physiological and the biochemical aspects of these varieties (information on physiological aspects see Clebsch et al., 2009). In a previous analysis we observed that Fepagro 26 and Irai have very different oxidative responses to O_3, suggesting that these varieties might present biochemical constitutive differences regarding their redox status and these differences could be associated with a higher tolerance or susceptibility to O_3 stress (see Caregnato et al., 2010). To further test whether the constitutive antioxidant biochemical differences between the varieties could account for their distinct responses to O_3 stress we decided to assess the following biochemical traits on the foliar tissue: (1) changes in the levels of ROS, (2) modifications on the immunocontent of the antioxidant enzyme catalase, (3) concentrations of non-enzymatic antioxidant (ascorbate and total thiol groups), and (4) and changes on the levels of the photosynthetic pigments chlorophylls *a* and *b*. In addition, *in situ* histochemical techniques were used to determine the type of ROS that was prevalently being produced during O_3 exposure.

2. Materials and methods

2.1. Plant material and growing conditions

Seeds from two different varieties of *Phaseolus vulgaris* L. (Fepagro 26 and Irai) developed by FEPAGRO-RS were germinated in 2 L pots containing 2:1:1 of coarse vermiculite, washed coarse sand and peat. The plants were grown under controlled conditions in a glasshouse, at an average temperature of 17.7 °C, and relative humidity of 88.6%. The plants were irrigated once a day with 100 mL of water. One day before O_3 treatment each plant was irrigated with 100 mL of Vitaplan Nutriverde 13-13-15 fertilizer (Nutriplan Products Company, Parana, Brazil). Eight days after the seeds were planted, 12 *P. vulgaris* seedlings from each variety were selected for the experiment. Six seedlings from each variety were subjected to O_3 exposure ($n = 6$), and six seedlings were used as control ($n = 6$).

2.2. Ozone exposure

Open top chambers were used for O_3 exposure experiments as previously reported by Caregnato et al. (2010) and Clebsch et al. (2009). Ozone fumigation was done using an ozonizer (ozone generator GHR150B, OZ Engenharia, Porto Alegre, Brazil) equipped with three O_3 production cells (corona effect) with a nominal production of 85 mg h^{-1} each. Fumigation was performed daily from 10:00 to 16:00 h during one week. Control plants were maintained in identical chambers in which normal air was introduced. Ozone monitoring was done on a 6 h d^{-1} basis (10:00–16:00 h) because this period is considered to be the peak of O_3 production found under ambient conditions. Ozone concentration inside the chambers was monitored by the iodometric method (APHA, 1992). Gases were sampled in a fresh washer of the impinger type, containing 75 mL of absorbent solution of KI 2% with the help of a gas sampler (LaMotte, model BD, Chestertown, USA) at a flow of 1.5 L min^{-1}. The sampling point was located in the central position of the chambers in relation to their diameters, and at the height of the unifoliolated primary leaves. The O_3 doses fumigated in each chamber (control and O_3) were calculated as the accumulated hourly O_3 exposure over a threshold concentration of 40 ppb (AOT_{40}) during daylight hours (10:00–16:00 h). Exposure index for O_3, i.e. AOT_{40}, was calculated and the mean value of O_3 concentration obtained on our experiments was 122.6 ppb h. During the experiment period each seedling was irrigated with 200 mL of tap water every day. At the end of the fumigation period, control and treated seedlings were harvested and the second leaf pair from each plant was sampled. Leaf samples were weighed and immediately stored at – 80 °C for biochemical analysis. The O_3 exposure experiments were performed on three independent sets, and the results presented are representative from one of the three biological replications. Results are presented as the average values from 6 seedlings for each treatment (control and O_3).

2.3. Determination of reactive oxygen species levels

About 50 mg of frozen tissue was used for the ROS level measurement assay. The assay was performed as described by Myhre et al. (2003). Leaf tissue was homogenized in 1 mL of Tris HCl 10 mM buffer (pH 7.2), and centrifuged at 14000*g* at 4 °C for 5 min. About 100 μL of this homogenate was mixed with 100 μL of 20 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Sigma Chemicals, St. Louis, MO, USA) and incubated in the dark for 10 min at room temperature. An aliquot from these homogenate was used to measure the sample fluorescence in fluorometer equipment (SpectraMax M5 model; Molecular Device, CA, USA). The results are expressed as relative fluorescence units (RFUs) per milligram of protein.

2.4. Histochemical staining

In situ histochemical assays were conducted for determining the nature of ROS as described by Thordal-Christensen et al. (1997) and Fryer et al. (2002) followed by minor modifications.

2.4.1. Superoxide radical detection

For detection of superoxide radical, 5 discs (diameter of 6.7 mm each) from all seedlings (O_3 and control) and from different leaves were incubated with 10 mM phosphate-buffered saline (pH 6.4) containing 0.01% nitroblue tetrazolium (NBT) during 60 min in the dark. Disc samples were illuminated for 2 h, when the reaction of NBT with superoxide anion stabilizes and the result appears as dark blue spots, which is a characteristic of blue formazan precipitates generation.

2.4.2. Hydrogen peroxide detection

To detect the H_2O_2 molecule, five leaf discs (diameter of 6.7 mm each) from all seedlings (O_3 and control) were immersed in diaminobenzidine (DAB) solution of 1 mg mL^{-1} (pH 3.8) during 8 h. As negative control, DAB solution was supplemented with 10 mM ascorbic acid, an antioxidant that prevents H_2O_2 generation.

Before visualization, disc samples from both NBT and DAB staining assays were bleached by immersing the samples in boiling solution of ethanol (96%) – glycerin (9:1 v/v) for 5 min to remove the chlorophyll. The destained discs were placed on a microscope slide and observed under a microscope (Nikon Eclipse TE300, Nikon, Melville, NU, USA), with a digital camera (Nikon Digital Camera DMX1200, Nikon, Melville, NU, USA) attached to it that was used to capture the images.

2.5. Determination of ascorbate concentration

Ascorbate concentration was assessed according to Keller and Schwager (1977). About 50 mg of frozen leaf tissue was homogenized in 1 mL of aqueous solution of EDTA-Na_2 (0.07%) and oxalic acid (0.5%). Samples were centrifuged at 14000 *g* during 30 min at 4 °C. An aliquot of supernatant was add 0.02% of 2,6-*Dichloroindophenol* Sodium Salt Dihydrate (DCPiP) (1:1 v/v). Samples were divided in two groups; to one of the groups were added 20 μL of ascorbic acid (1%). This group was considered the positive control. The absorbance of each sample was measured at $\lambda = 520$ nm in a micro-plate reader and the difference in the absorbance between the two groups (group without ascorbic acid – group with ascorbic acid) were used to quantify the total amount of ascorbate. Values are expressed as mg of ascorbate per milligram of protein.

2.6. Total reduced thiol content

Leaf samples were analyzed for their content of total reduced thiol, which is considered an estimation of intracellular redox balance alterations. As previously described by Ellman (1959), an aliquot of the sample (30–80 μg protein) was diluted in 10 mM phosphate-buffered saline (pH 7.4) Then, 0.01 M 5,5-dithiobis-2-nitrobenzoic acid (DTNB) dissolved in ethanol, was added. After 60 minutes an intense yellow color was developed and samples were read in a spectrophotometer at 412 nm. Values are expressed as nmol of thiol groups (SH) per milligram of protein.

2.7. Catalase immunoblotting

To perform immunoblot experiments leaf samples were homogenized in Laemmli-sample buffer (62.5 mM Tris–HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol) and equal amounts of protein (60 μg/lane) were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) and electroblotted onto polyvinyledilene difluorife (PVDF) membranes. Protein loading and electroblotting efficiency were verified through Ponceau S staining, and the membrane was blocked in Tween–Tris buffered saline (TTBS:100 mM Tris–HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20) containing 0.5% albumin and incubated overnight with the primary antibody to be tested. The membrane was washed and incubated with Horseradish peroxidasecoupled anti-rabbit IgG antibodies, and the immunoreactivity was detected by enhanced chemiluminescence using the West Pico detection Kit (Pierce, Rockford, IL, USA). Densitometric analysis of the X-ray films exposed to the chemiluminescence was performed with the IMAGE J® software. Blots were developed to be linear in the range used for densitometry. All results are expressed as relative CAT units, and Comassie-blue staining was used as the gel internal loading control.

2.8. Chlorophyll content

Chlorophylls *a* and *b* content was measured by UV–VIS Spectroscopy. Ten foliar discs of 6.7 mm in diameter were incubated in 10 mL of ethanol 96% during 1 week in darkness. The absorbance of leaf pigment extracted in the ethanol was measured at 649 and 665 nm. Levels of chlorophylls *a* and *b*, total chlorophylls ($a + b$) and chlorophyll ratio (a/b) were calculated according to extinction coefficients and equations reported by Lichtenthaler (1987). Data from the absorbance were averaged and chlorophyll content was expressed on basis of final volume of ethanol and the dry weight of leaf discs after the period of incubation. Results are expressed as mg of chlorophyll per gram of dry weight.

2.9. Protein content

Protein content was measured spectrophotometrically at 595 nm by the protein–dye binding method of Bradford (1976), using bovine serum albumin as standard.

2.10. Statistical analysis

Data shown in figures and tables are means ± SEM for each variety. Differences between the varieties were analyzed employing two-way analysis of variance (ANOVA) and the student *t*-test. Statistically significant differences found between controls and ozone exposed on the ANOVA test were assessed via Bonferroni post-hoc test, with a significant level set at $p < 0.05$. The relationship between chlorophylls a/b ratio and protein concentration in leaf tissue were assessed using linear bivariate regression analysis within GraphPad software version 5.01 (Graphpad Software Inc., 2007).

3. Results

3.1. Reactive oxygen species generation and histochemical staining

The two *P. vulgaris* varieties showed different patterns of ROS production in leaf tissue when growing under the presence of O_3. Fepagro 26 exposed seedlings presented lower constitutive levels of ROS content, but significantly increased the foliar ROS concentration in response to O_3 treatment. After the exposure period of 1 week, ROS levels on Fepagro 26 leaves were 1.8-fold higher than control plants. Conversely, no significant changes on Irai leaf ROS levels could be observed after the same exposure time period (Fig. 1). However, the endogenous levels of ROS on Irai leaves were 1.2-times higher than Fepagro 26 ($p < 0.0001$).

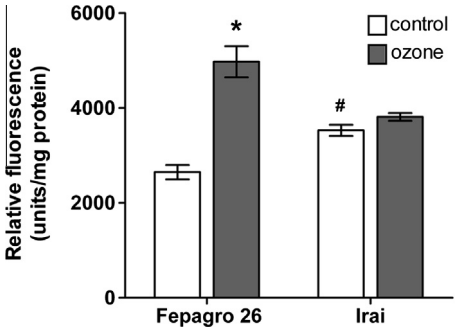

Fig. 1. Reactive oxygen species levels on control and O_3-exposed leaves from Fepagro 26 and Irai seedlings. Values represent mean ± SEM ($n = 6$) of three independent experiments. * Indicate difference from control ($p < 0.05$); # indicate difference between varieties ($p < 0.05$) as analyzed by ANOVA.

To assess the type of ROS that was prevalently being produced during the O_3 exposition we used two *in situ* histochemical techniques. Diaminobenzidine (DAB) reacts with H_2O_2 to form reddish-brown deposits that can be examined with a light microscope. Image analyses showed that Fepagro 26 fumigated leaves present more intense spots for DAB staining than control leaves (Fig. 2, arrows). On the other hand, when analyzing Irai leaves no substantial changes in the amount of DAB precipitates formed during the same time period could be noticed. When comparing Irai control and exposed leaves the same pattern of brow spots intensity could be observed. To ensure that the results we were seeing were really from the reaction between H_2O_2 and DAB, we tested the samples with a positive control. Ascorbate (10 mM) was added to the DAB staining solution and the incubation of leaf discs was performed. Almost all DAB deposits were completely reduced on these samples, indicating that DAB staining was actually due to local differences in H_2O_2 generation (data not shown).

The production of superoxide radical was imaged in leaves infiltrated with NBT, which specifically reacts with superoxide anions resulting in purple formazan deposits, which can easily be visualized in the microscopy. Within 1 week of treatment visible heavier staining spots were observed on Fepagro 26 exposed leaves, indicating that superoxide radical production was higher on these leaves than control (Fig. 2, arrows). When comparing exposed and non-exposed Irai leaves, we observed that generation of purple formazam precipitates occurred on both groups, and when comparing exposed Irai and Fepagro 26 leaves we could notice that a lighter staining was found on Irai leaves.

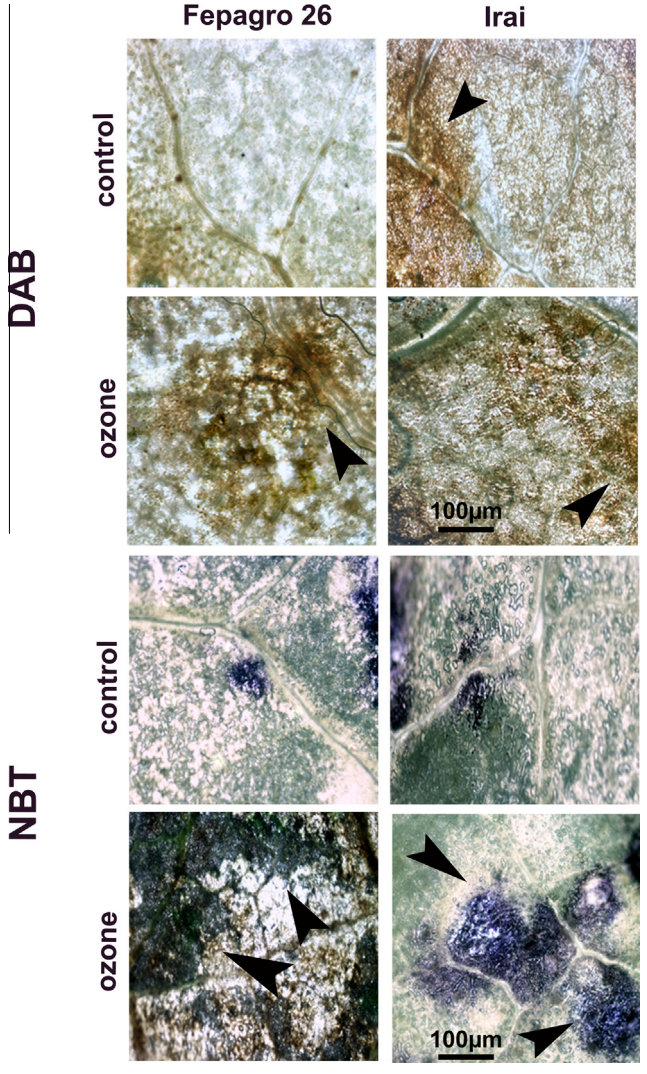

Fig. 2. *In situ* localization of O_2^- and H_2O_2 respectively by NBT and DAB staining in control and O_3-exposed leaves from Fepagro 26 and Irai seedlings. Arrows are indicating the H_2O_2 accumulation areas (brown-stained spots), and O_2^- accumulation areas (purple spots) (magnification 10×). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Non-enzymatic antioxidant defense system

3.2.1. Ascorbate levels

Ozone sensitivity is generally correlated with the oxidative status of AsA on leaf tissue, and the antioxidant role played by AsA molecule is considered to be strictly dependent on the cell ability to maintain it in a reduced state (Castagna and Ranieri, 2009; Conklin and Barth, 2004). Comparing the AsA levels of Irai and Fepagro 26 leaves it was evident that the endogenous levels of AsA on Fepagro 26 are significantly lower than Irai ($p < 0.0001$), in fact AsA concentration of Fepagro 26 are 1.26 times lower than Irai (Fig. 3a). After 1 week of O_3 treatment the AsA pool in Fepagro 26 leaves was changed, and a significant increase of nearly 50% on the AsA concentration was recorded on exposed Fepagro 26 plants. On the opposite, AsA levels on Irai leaves were not changed in response to O_3 exposure (Fig. 3a).

3.2.2. Total reduced thiol content

The physiological significance of thiol metabolism in plants is specially related to sulfur assimilation and antioxidant defense. Here we could notice that significant differences on the physiological levels of total reduced thiol content when comparing the two *P. vulgaris* varieties. Leaves from Fepagro 26 had a baseline level of total reduced thiol content 4.2-fold higher than Irai

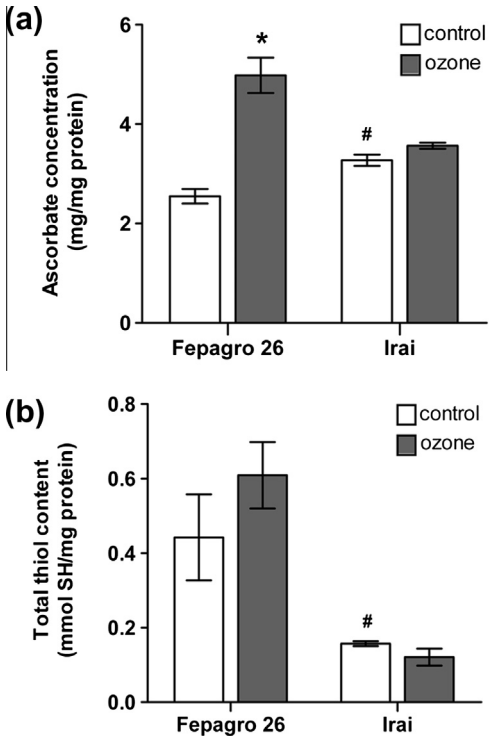

Fig. 3. (a) Ascorbate concentration and (b) total thiol content on control and O_3-exposed leaves from Fepagro 26 and Irai seedlings. Values represent mean ± SEM ($n = 6$) of three independent experiments. * Indicate difference from control ($p < 0.05$); # indicate difference between varieties ($p < 0.05$) as analyzed by ANOVA.

($p = 0.0007$). After 1 week of O_3 fumigation no significant changes on the levels of total thiol concentration were observed neither for Fepagro 26 nor Irai leaves (Fig. 3b).

3.3. Catalase immunoblotting analysis

To better understand how O_3 exposure was affecting the leaves enzymatic antioxidant apparatus at the level of enzyme amount, we decided to perform a quantitative analysis of the catalase enzyme. As previously reported, the two varieties have a very different response of CAT activation when exposed to O_3 (Caregnato et al., 2010). Here we demonstrated that the differences observed on CAT activity are based on the amount of CAT protein. Irai leaves showed a significant lower trend on the baseline CAT immunocontent than Fepagro 26 ($p = 0.02$). Catalase immunocontent on Fepagro 26 leaf tissue was 1.66 times higher than Irai leaves. But differently from Fepagro 26, Irai seedlings challenged with O_3 were capable of increasing the amount of CAT protein. Catalase immunocontent on treated Irai plants were found to be 2 times higher when compared to control Irai plants ($p = 0.01$) (Fig. 4).

3.4. Photosynthetic pigments content

The levels of photosynthetic pigments were differently affected by O_3 exposure on each *P. vulgaris* variety (Table 1). As suggested by the loss of leaves green color, a significant declining trend under O_3 exposure was observed in both chlorophylls *a* and *b* (53% and 40%, respectively) on O_3 fumigated Fepagro 26 leaves. Total chlorophyll (Chl *a* + Chl *b*) content was also affected by O_3, and a significant reduction of approximately 50% of total Chl was recorded in the O_3-exposed Fepagro 26. Furthermore, a significant decrease was also observed on the Chl *a*/*b* ratio, which indicates that Chl *a* levels was probably more affected by the O_3 treatment than Chl *b*. On the opposite, no modifications in the levels of the foliar pigmentation of O_3-treated Irai leaves could be observed, indicating that chlorophyll pigments levels were not affected when this cultivar is growing under the presence of elevated O_3 concentration. In this variety neither Chl *a* nor Chl *b* amounts were found to be modified by O_3 treatment, and thus no differences could be assessed either on total Chl content or on the Chl *a*/*b* ratio.

3.5. Relationship between chlorophyll and protein concentration

Levels of total protein content were significantly decreased in response to O_3 treatment in both varieties. However, the reduction on protein content was significantly higher in Fepagro 26 (approximately 50%), when compared to total protein content loss found on Irai plants (5.6%) ($p < 0.05$) (Table 1). In order to assess if there was a significant relationship between the chlorophyll content and total protein concentration we performed a linear regression analysis. To the test this relationship we choose to use the ratio of photosynthetic pigments, chlorophylls *a* and *b*, once their concentration are tightly regulated in the plant photosynthesis apparatus and their proportional changes may affect the entire photosynthesis process. We found a significant positive linear relationship between the protein content and Chl *a*/*b* ratio only for Fepagro 26 seedlings (Fig. 5a). Regression analysis results indicate thus that a decrease in the Chl *a*/*b* ratio is significantly associated with a decrease in total protein concentration when Fepagro 26 plants grown under the presence of toxic O_3 concentrations ($r^2 = 0.87$; $p < 0.0005$; $F = 49.63$). Since no differences on chlorophylls *a* and *b* content could be found on fumigated Irai leaves, no association between this parameter and protein content were observed ($r^2 = 0.48$; $p = 0.11$; $F = 3.07$) (Fig. 5b).

4. Discussion

The present study reveals that the antioxidant capacity on leaf tissue of two Brazilian *P. vulgaris* varieties exposed to high O_3 concentration during the period of 1 week was significantly affected. Our results showed that integrated biochemical mechanisms related to a better capacity to counterbalance the O_3-mediated oxidative process are behind the differences on the O_3-susceptibility observed between the two studied bean varieties. Not differently from the most up-to-date studies we demonstrated here that the detrimental effects of O_3 on plant metabolism occur due to its high oxidative power and ability to be converted into different ROS in the leaf internal space. However, as plant species possess a wide range of responses to cope with O_3-induced oxidative stress and, as suggested by other experiments with different crops worldwide, variation on these responses can be attributed to innate and inducible antioxidant biochemical features that are distinct among plant species and varieties (Burkey et al., 2005; Scebba et al., 2006; Tosti et al., 2006; Matyssek et al., 2008). Differential sensitivity to O_3 exhibited by different varieties within a plant species has been proven to be especially dependent on the efficiency of ROS scavenging system to maintain the cellular redox steady-state of leaf tissue (Puckette et al., 2007; Castagna and Ranieri, 2009; Giacomo et al., 2010).

The evaluation of ROS levels through DCF analysis revealed that Irai variety present higher constitutive levels of ROS than Fepagro 26 (Fig. 1). Ozone exposure did not alter the ROS generation on Irai

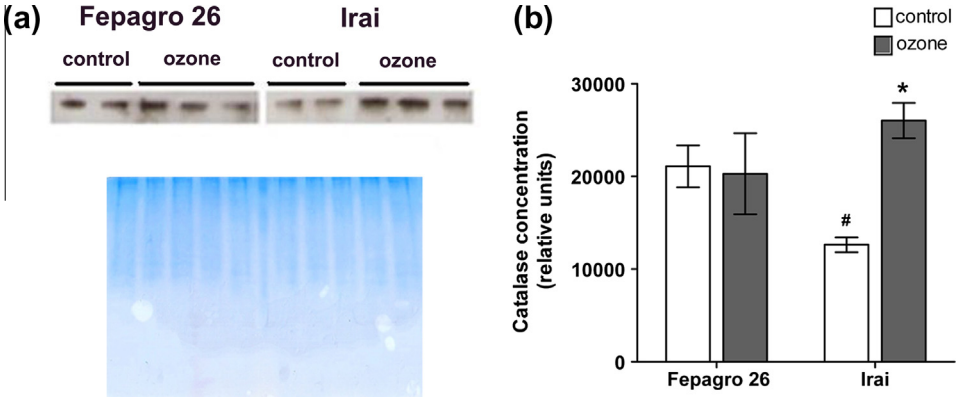

Fig. 4. (a) Catalase immunocontent on control and O_3-exposed leaves from Fepagro 26 and Irai seedlings and loading control with Comassie Blue staining. (b) Catalase immunocontent expressed as relative units. Values represent mean ± SEM ($n = 3$) of three independent experiments. * Indicate difference from control ($p < 0.05$); # indicate difference between varieties ($p < 0.05$) as analyzed by student *t*-test.

Table 1
Chlorophyll a and b content, and protein concentration on control and O_3-exposed *Phaseolus vulgaris* seedlings. Values represent mean ± SEM ($n = 6$) of three independent experiments.

	Fepagro 26			Irai		
	Control	O_3 treated	*p* Value	Control	O_3 treated	*p* Value
Chlorophyll a (mg g^{-1})	7.7 ± 0.41	3.6 ± 0.31*	<0.0001	7.8 ± 0.71	6.5 ± 0.44	ns
Chlorophyll b (mg g^{-1})	3.5 ± 0.20	2.1 ± 0.17*	<0.001	3.7 ± 0.34	3.2 ± 0.14	ns
Total chlorophyll (mg g^{-1})	11.3 ± 0.59	5.7 ± 0.49*	<0.001	11.62 ± 1.06	9.8 ± 0.60	ns
Chlorophyll *a*/*b* ratio	2.1 ± 0.040	1.7 ± 0.062*	<0.0005	2.1 ± 0.030	2.0 ± 0.04	ns
Protein (μg mL^{-1})	3227.9 ± 183	1722.1 ± 130*	<0.001	2353.6 ± 77	2219.5 ± 48*	<0.05

ns – Indicates no significant differences between control and exposed leaves as analyzed by ANOVA.
* Indicate difference between control and exposed leaves ($p < 0.05$).

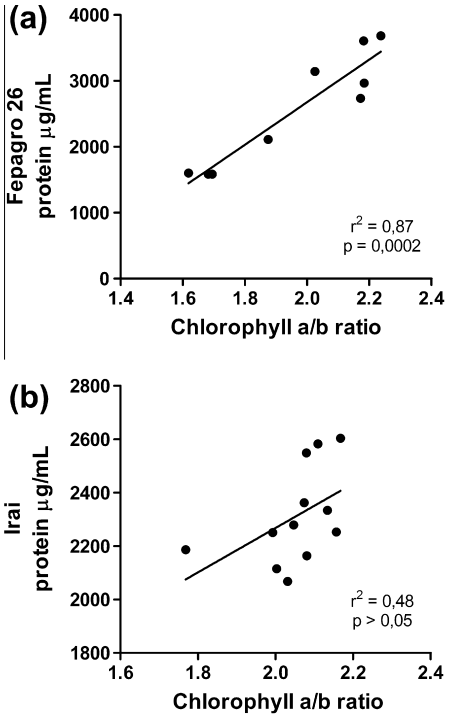

Fig. 5. Relationship between chlorophyll content and total protein levels on control and O_3-exposed leaves from (a) Fepagro 26 and (b) Irai. Values represent mean ± SEM ($n = 6$) of three independent experiments. Data were analyzed using linear bivariate regression analysis. The significance of the *p*-values are shown, and a significant relationship was considered for a $p < 0.05$.

leaves, but significantly increased the ROS production in Fepagro 26 plants. Using histochemical *in situ* localization techniques we could detect and visualize the two major ROS, superoxide radical and H_2O_2, on leaf tissue from both varieties (Fig. 2). On Fepagro 26 leaf samples we observed that both superoxide radical and H_2O_2 accounted for the significant enhancement on ROS levels after O_3 treatment. The strong staining pattern observed after the reaction of superoxide radical with NBT on O_3-exposed Fepagro 26 leaves suggest that the levels of this ROS was pronouncedly increased on this bean variety. With the *in situ* localization techniques we were also able to notice that Irai control leaves showed stronger stained spots for DAB reaction suggesting that endogenous levels of H_2O_2 on this variety are constitutively higher than those levels found on Fepagro 26 control leaves (Fig. 2). Previous studies have demonstrated that distinct patterns of ROS accumulation occurs in differentially O_3-sensitive plants as shown for tobacco (*Nicotiana tabacum* L. cv Bel W3) and clover plants (*Trifolium repens* L.) (Pasqualini et al., 2003; Scebba et al., 2006). In sensitive varieties both superoxide radical and H_2O_2 accumulate in leaf margins following O_3 exposure, and the sites of ROS generation (particularly H_2O_2) has been correlated with the appearance of leaf injury signals, such as necrotic lesions (Di Baccio et al., 2008; for review see Castagna and Ranieri, 2009). Furthermore, Guidi et al. (2010) when comparing the responses of two *P. vulgaris* cultivars to acute O_3 treatment (165 nL L^{-1}) showed that after 3 h of exposure the levels of H_2O_2 accumulated to a markedly greater extent in the leaves of the sensitive cultivar Cannellino than in tolerant Top Crop, whereas the H_2O_2 deposits were only become detectable for Top Crop after 24 h from the beginning of the fumigation treatment. In the O_3-sensitive cultivar Cannellino this early accumulation of H_2O_2 was found to be correlated to the almost complete disruption of cell structure, and irreversible damages to the photosynthetic apparatus. In addition, interesting inverse correlation between the levels of ROS in control and O_3 sensitivity plants were reported for 38 different accessions of *Medicago truncatula* fumigated with 300 nmol mol^{-1} of O_3 for 6 h. Accessions with higher levels of endogenous ROS were more tolerant compared to accessions with lower ROS levels, suggesting that plants with elevated ROS levels may be 'intrinsically primed' to tolerate extreme oxidative stress, a fact that strengths the role of ROS as important signaling molecules during normal growth and stress conditions (Puckette et al., 2007).

Nevertheless, the degree of sensitivity of a plant to O_3 not only depends on the quantity of gaseous molecules entering the leaf and that are subsequently converted to ROS. Over the past years, several studies have pointed out that the main level of O_3 defense relies both on the existing content of cellular antioxidants (e.g. ascorbate and glutathione) and the intensity of the detoxifying pathways that are responsible for regenerating these metabolites (Calatayud et al., 2001; Dizengremel et al., 2008, 2009; Luwe et al., 1993). The protective role of AsA as ROS-scavenger was first supported by the enhanced O_3-sensitivity shown by *Arabidopsis thaliana* mutants deficient in AsA synthesis (Conklin et al., 1996). Even so, the relationship between O_3 sensitivity and apoplastic AsA concentration remains controversial and some studies have postulated that elevated apoplastic AsA levels cannot always be sufficient to render a plant tolerant to O_3 (Ranieri et al., 1999; D'Haese et al., 2005; Di Baccio et al., 2008). Here we found that Irai plants have higher constitutive AsA levels than Fepagro 26, and O_3 exposure does not modify the levels of this antioxidant molecule on Irai leaf tissue (Fig. 3a). Although it is well-known that apoplastic AsA is quickly oxidized when in presence of O_3 and ROS, our results may indicate that Irai plants are capable to maintain a higher AsA-mediated antioxidant capacity, a feature that is probably related to the ability of the foliar cells to keep both extra and intracellular supply of reduced AsA at levels that are effective enough to detoxify ROS generated under the presence of O_3. Conversely, Fepagro 26 baseline levels of AsA were significantly lower, and when this variety was fumigated with O_3 the levels of foliar AsA were significantly enhanced. These findings suggest that O_3 exposure of Fepagro 26 plants might stimulate AsA production and/or its

movement from the intracellular to apoplastic space in attempt to increase the amount of ROS that can be scavenged by the cells. The rate of AsA regeneration controls the O_3 influx to leaf interior, which means that non-detoxified O_3 molecules are subsequently free to promote its oxidative action, especially trough its conversion to ROS in the leaf apoplast (Smirnoff, 1996; van Hove et al., 2001; Di Baccio et al., 2008). In spite of the fact that O_3-treated Fepagro 26 increases the concentration of AsA, its antioxidant capacity to respond O_3 influx is probably less effective than that of Irai. Together with the results from foliar ROS accumulation we can infer that the AsA antioxidant capacity per unit of O_3 flux is decreased in Fepagro 26, and thus more O_3 molecules are free to being decomposed into ROS (H_2O_2 and superoxide radical) inside the foliar tissue.

The antioxidant role played by AsA is, however, strictly dependent on the cell ability to maintain it in a reduced state which occurs trough the action of AsA–GSH cycle (Smirnoff, 1996; Noctor and Foyer, 1998; Di Baccio et al., 2008). Using high O_3 concentrations (300 ppb) Luwe et al. (1993) observed a time-dependent relationship between oxidation of both extracellular AsA and intracellular GSH pool, while the cellular AsA redox state was unaltered during fumigation. As reported by numerous other studies, AsA regeneration is tightly coupled to GSH within the cell and transport activity was responsible for replenish the reduced apoplastic AsA pool (for review see Horemans et al., 2000b and Smirnoff, 1996). Thus, trough a GSH-dependent regeneration mechanism, the extracellular AsA oxidation is supposed to affect the intracellular thiol signature. Analysis on the levels of total reduced thiol indicated that in both varieties the antioxidant thiol pool was unaffected by O_3 exposure (Fig. 3b); nonetheless, there was a significant difference in the endogenous pool size between Irai and Fepagro 26, being the values for total reduced thiol content on Fepagro 26 leaves 4.2 times higher than Irai. Unlike the apoplasm, the cytoplasm is a cellular compartment where innumerous molecules involved in different metabolic pathways are localized. Modifications on the intracellular redox balance can directly modify proteins structures, and changes in thiol-redox state can modulate enzymes activity by altering active site residues required for catalysis and/or ligand binding, oligomerization, and cellular localization (Paget and Buttner, 2003; Yi et al., 2010). Thus, besides GSH other reducing co-factors for several enzymes involved in ROS detoxification are also conjugate to proteins to avoid their oxidation, and this process is catalyzed by enzymes such as glutaredoxins, thioredoxins and peroxiredoxins (Foyer et al., 1997; Halliwell, 2006) . Here we analyzed leaf total reduced thiol content, not only the GSH levels, and maybe this why we have not observed a positive relationship between the levels of total reduced thiol and AsA during O_3 detoxification process on Fepagro 26 plants. Based on recently proteomic studies that support that thiol-based regulation play a critical role in plant responses to O_3 exposure (Gillespie et al., 2011; Galant et al., 2012), we can suggest that in an effort to avoid deleterious oxidative changes on proteins, Fepagro 26 leaf cells try to maintain the levels of total reduced thiol as a mechanism of redox-protection of enzymes involved in major metabolic processes.

In addition to the non-enzimatic antioxidant components, the balance of antioxidant enzymes activities is crucial for suppressing the toxic effects of elevated ROS levels (Apel and Hirt, 2004). Modulation of the major antioxidant enzymes activities (SOD, CAT, APX, GR) have been observed in different O_3 exposure experiments (Pell et al., 1999; Calatayud et al., 2003; Heath, 2007). The changes on enzymes activities are often linked to parallel changes in gene expression in the intracellular compartment (Sharma and Davis, 1994; Willekens et al., 1994; Pellinen et al., 2002; Pasqualini et al., 2007; Torres et al., 2007). On a previous work, when comparing the results for CAT activity, we observed that the two varieties had a very different pattern of CAT stimulation in response to O_3 fumigation. Irai seedlings significantly increased CAT activity after O_3 exposure, whereas Fepagro 26 did not change the enzyme activity when challenged with the same O_3 concentrations (AOT_{40}=212 - ppb h, during one week) (Caregnato et al., 2010). Trough western blot analysis we demonstrated that, even though Irai leaves have lower endogenous levels of CAT (Fig. 4), when this variety is subjected to O_3 treatment the concentration of the antioxidant enzyme is significantly increased. Altogether, our results indicate that in Irai leaves the increment on CAT activity elicited by the presence of O_3 is probably a consequence of the increased concentration of the enzyme found on the intracellular space, a fact that might improve the antioxidant capacity of Irai foliar cells. Catalase in the one of the most important enzymes needed for H_2O_2 detoxification (Willekens et al., 1995). Plants contain several types of H_2O_2-degrading enzymes (peroxidases), however CAT are unique as it do not require cellular reducing equivalents to convert H_2O_2 to water (Sharma et al., 2012). The most important reducing substrate used for peroxidases is the AsA (Mehlhorn et al., 1996), an antioxidant molecule that is easily oxidized under stressful conditions such as O_3 exposure. Stress analysis on transgenic CAT-deficient *Arabidopsis thaliana* revealed that reduced peroxissomal CAT activity increased sensitivity towards O_3 and triggers cell death by photorespiratory H_2O_2 (Vandenabeele et al., 2004). Transgenic tobacco plants with approximately 10% of wild-type CAT activity showed accumulation of oxidized GSH (GSSG) and a 4-fold decrease in AsA; moreover, CAT deficiency renders tobacco transgenic plants markedly more vulnerable to O_3 stress (Willekens et al., 1997). In sum, in a pro-oxidant environment an effective CAT action is critical for maintaining the redox balance to avoid further oxidative stress.

It is known that one of the main detrimental effects of O_3 that occur at leaf subcellular level is the impairment of the photosynthesis process (Pell et al., 1994). Ozone reduction in net photosynthesis is attributed to be a consequence of degradation of both structural and functional components of the chloroplasts (Pääkkönen et al., 1996). Trough the oxidation of membrane lipids, O_3 and its derived ROS can cause the disruption of biological membranes, leading to irreversible modifications of chloroplast components, such as the tylakoids (Calatayud et al., 2001, 2003 Anderson et al., 2003). Tylakoids disintegration results in the loss of membrane attached chlorophyll molecules, which in free solution are prone to be oxidatively damaged by ROS present in the intracellular environment (Sakaki et al., 1983; Ranieri et al., 2001). Moreover, recent analyses of transcriptome changes in O_3-sensitive and resistant *Medicago truncatula* accessions identified that genes involved in chlorophyll biosynthesis were down regulated after 6 h post-fumigation on sensitive accession Jemalong (Puckette et al., 2008). When analyzing leaf chlorophyll content we found that chlorophylls *a* and *b*, total chlorophyll and the ratio of Chl *a*/*b* levels on O_3-exposed Fepagro 26 plants were significantly reduced, a change that was not observed on Irai leaves (Table 1). Considering that Fepagro 26 leaves are unable to properly detoxify the ROS, we can suggest that chlorophyll loss can be either associated to increased chlorophyll oxidation or to an altered regulation of pigments biosynthesis. A comparative study with 12 bean varieties demonstrated that, after 75–135 min of O_3 exposure, a high stomatal conductance was accompanied by chlorophyll loss and/or inhibition of photosynthesis was a common feature of sensitive varieties (Guzy and Heath, 1993). Furthermore, Leitao et al. (2008) observed that *P. vulgaris* plants exposed to non-filtered ambient air supplied with 80 nL L^{-1} of O_3 had a loss of 43% on total chlorophyll content. Similarly to our results, reductions in both Chl *a* and *b* were evident, and as underlined by the authors, a similar trend of Chl *a*/*b* ratio reduction indicate that Chl *a* tended to be decreased a little more by O_3 than Chl *b*.

Using correlation analysis we could show that when chlorophylls *a*/*b* ratio is decreased on Fepagro 26 exposed plants, the amount of total protein is also reduced. Such significant relationship was not observed for Irai. This result may indicate that ROS accumulation is leading to chlorophyll loss, which affects the photosynthetic process and the biomass acquisition of Fepagro 26 exposed plants. Clebsch et al. (2009) studying different subtropical bean varieties, including Fepagro 26 and Irai, observed that O_3 treatment decrease the carbon net assimilation on all studied varieties, but Irai. Carboxylation efficiency, which is considered to be the main factor involved in photosynthesis performance (Pell et al., 1994), was found to be significantly reduced in Fepagro 26 seedlings. Disruption of photosynthetic apparatus might be responsible for reduced carboxylation efficiency by the Calvin cycle enzymes, which finally result in growth impairment on this variety. Experimental studies have recently described that high O_3-mediated impairment on growth and yield of two important Indian rice cultivars (*Oryza sativa* L. cvs Malviya dhan 36 and Shivani) are associated to reductions of plant height, leaf area, total chlorophyll content, photosynthetic rate, stomatal conductance, and chlorophyll fluorescence (Sarkar and Agrawal, 2012). In addition, studies done with a particular O_3-sensitive crop, soybean (*Glycine max*), demonstrated that O_3 exposure negatively impacts the plants efficiency to convert solar energy into biomass energy by reducing photosynthetic efficiency and by increasing respiratory costs, which would contribute to yield loss (Betzelberger et al., 2012).

In fact, numerous studies throughout the world that performed similar experiments with a wide range of crops exposed to O_3 have showed that, on sensitive plants, the changes on redox-related biochemical parameters are related with yield losses and, recently, with altered grain quality and nutritional traits (Flowers et al., 2007; Feng and Kobayashi, 2009; Iriti et al., 2009; Sarkar and Agrawal, 2010; Wang et al., 2012). Considering that the risk of the negative effects of O_3 on crop productivity has been creating the need for new crop varieties that are better adapted to O_3 stress, it is of particular importance to further study how the O_3-induced changes on the redox status on the sensitive Fepagro 26 can be associated to modifications on grain quality and yield on this variety. In addition, *P. vulgaris* L. is considered by some authors to be one of the most sensitive agricultural crops to O_3 toxic effects, and experiments to better predict the impacts of increasing O_3 levels are urgently needed. Thus, it must be noted that improved understanding of the exposure–response mechanisms underlying O_3 sensitivity/tolerance are still necessary for major Brazilian bean varieties.

5. Conclusions

Based on our results we can suggest that Fepagro 26 is more likely to suffer the negative impacts of high O_3 levels than Irai, which seems to be a more robust variety. The greater sensitivity of Fepagro 26 variety to O_3 stress seems to be associated with an increased ROS production (superoxide radical and H_2O_2) which creates a pro-oxidative environment inside the cells. Apparently, both enzymatic (CAT) and non-enzymatic (AsA) leaf antioxidants are unable to maintain the intracellular redox homeostasis, which leads to oxidative damages to structural molecules such as the membrane lipids (see Caregnato et al., 2010). Disruption of membrane integrity can cause photosynthetic pigments loss (chlorophylls *a* and *b*), which reduces the photosynthesis rate and affects the biomass acquisition of Fepagro 26 plants (protein levels reduction). On the opposite, Irai presents higher endogenous levels of ROS, and under the presence of O_3 its antioxidant apparatus is stimulated to avoid oxidative stress. Similarly to the conclusions achieved by Guidi et al. (2010), the O_3-tolerance in bean depends more on the specific potential ability of each cultivar than the stress-induced physiological and biochemical adjustments to avoid and counter stress-induced oxidative damage. Although the parameters assessed in this study could arise as a consequence of other environmental stresses, our data provide reliable results concerning some of the major biochemical differences that are behind the responses of the two subtropical *P. vulgaris* varieties to the O_3 exposure. To better characterize these bean varieties according to their O_3 susceptibility an assessment of dose dependent sensitivity with respect to growth, plant yield parameters and grain quality should be further conducted.

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